



Technical note: Assessing potential hydrolytic enzyme activities on frozen samples as a tool to bridge microbial rate measurements with omics analysis

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Abstract. Extracellular hydrolytic enzymes play a central role in marine carbon and nutrient cycling. Their activities are typically measured immediately after sample collection, limiting integration with omics analyses conducted on frozen samples. We assessed the effects of one month of storage at -20°C and -80°C on potential activities of leucine aminopeptidase (LAP), β -glucosidase (β -glu), N-acetyl- β -D-glucosaminidase (NAG), and alkaline phosphatase (AP) in estuarine and offshore samples from the Gulf of Maine. Activities of LAP, β -glu, and NAG were generally well preserved on frozen filters, particularly at -80°C. In contrast, AP showed greater sensitivity to freezing, especially in offshore particle-associated samples. All enzymes exhibited greater activity losses in frozen whole water samples than on filters. These results demonstrate that frozen filters, particularly when stored at -80°C, can preserve several ecologically relevant extracellular enzyme activities and provide a practical approach to link enzyme assays with omics measurements on the same samples.

1 Introduction

Marine microorganisms sustain ocean food webs and play a central role in the cycling of carbon, nitrogen, phosphorus, sulfur, and other key elements. Their contribution to ecosystem functioning depends not only on their abundance and diversity, but also on the rates at which they process and transform these elements. Combining measurements of microbial diversity with biogeochemical rate measurements is a major goal in microbial oceanography and provides the mechanistic foundation needed to improve ocean biogeochemical models (Tagliabue 2023).

A promising tool for linking microbial diversity measurements with biogeochemical rate measurements is assessing hydrolytic enzyme activities. Hydrolytic enzymes play a central role in biogeochemical cycles by initiating the microbial degradation of organic matter and thereby regulating the flow of carbon, nutrients, and energy through aquatic food webs (Chrost 1991; Arnosti 2011). Activities of extracellular enzymes are commonly measured in incubation assays using chromogenic or fluorogenic substrate analogs that serve as proxies for naturally occurring organic compounds. Among these approaches, the fluorometric assay developed by Hoppe (1983) has become widely adopted in aquatic and terrestrial microbial ecology. The



30 assay uses commercially available coumarin-labeled substrates that release a fluorescent product upon enzymatic cleavage. It is relatively inexpensive, sensitive, and readily adaptable to high-throughput measurements using microtiter plates, making it well suited for large-scale studies aimed at linking microbial function with community composition.

A logical next step is to conduct enzyme assays and omics analyses on the same environmental samples, such as particulate material collected on polycarbonate filters (D'Ambrosio et al. 2014). Because omics analyses are routinely performed on filters

35 stored frozen prior to analysis, we tested whether potential hydrolytic enzyme activities are preserved on frozen filters and can be measured after storage. Demonstrating such preservation would provide a practical framework for integrating enzyme activity measurements with metagenomic, metatranscriptomic, and metaproteomic analyses.

2 Materials and methods

2.1 Sampling and water processing

40 Water samples were collected from an estuarine and an offshore site in the Gulf of Maine (Table 1). Estuarine water was sampled at high tide by submerging replicate 5-L carboys below the surface at the Jackson's Landing boathouse pier in Durham, New Hampshire, about 1 mile from the University of New Hampshire campus laboratory. Water from the offshore site was taken in Wilkinson Basin, approximately 40 miles off the coast of New Hampshire. Water was collected from 2 m depth using Niskin bottles mounted on a CTD rosette deployed from the R/V Gulf Challenger.

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Table 1. Sampling locations, environmental conditions, and sample types collected for enzyme activity measurements. Water temperature and salinity were measured at the time of sampling. Numbers in parentheses indicate the total number of replicate samples analysed.

Sampling site	Sampling date	Lat/long	Water temp, °C	Salinity, PSU	Sample ID (# of replicates)
Estuary	Nov 13, 2024	43.134° N 70.910° W	8	31	whole water (9); filters (9)
Offshore	June 18, 2025	42.861° N 69.861° W	15	31.5	filters (3)

50 Samples from both sites were processed within 1 h of collection, either in the campus laboratory (estuarine samples) or in the shipboard laboratory (offshore samples). Water was filtered onto replicate 0.4-µm Isopore membrane filters (Millipore Sigma) under low vacuum (500 mL per filter). Nine filters were prepared from the estuarine samples (three filters from each of three replicate carboys), whereas operational constraints aboard the vessel limited offshore collections to three filters. Filters were placed individually into cryovials and stored at either -20°C or -80°C (estuarine: 3 filters per temperature treatment; offshore:

55 1 filter per temperature treatment). The remaining filters (estuarine: 3 filters; offshore: 1 filter) were processed immediately for enzyme activity measurements and served as initial activity controls. For the estuarine site, we also stored unfiltered water samples at -20°C and -80°C to assess the effects of freezer storage on enzyme activities in bulk seawater (hereafter referred to as whole water).



60 2.2 Enzyme assays

To measure particle-associated enzyme activities, filters were transferred to individual 15-mL centrifuge tubes (Corning Falcon) containing 10 mL artificial seawater (ASW). Artificial seawater was prepared from Millipore Sigma Sea Salts dissolved in Milli-Q water (pH 8.1) and sterile-filtered through 0.2- μ m cellulose acetate syringe filters (Millipore Sigma). Tubes were vortexed for 30 s to resuspend particles from the filter into the ASW.

65 Enzyme assays were conducted using particle suspensions, whole water samples, and ASW controls lacking particles following the protocol by Hoppe (1983). Incubations were performed in replicates ($n = 3$) using black 96-well microplates (Corning Flat Bottom Black plates) preloaded with the following fluorogenic substrate analogs: 4-methylumbelliferyl (MUF)- β -D-glucopyranoside (β -glu), MUF-phosphate (AP), MUF-N-acetyl- β -D-glucosaminide (NAG), and L-leucine-7-amido-4-methylcoumarin hydrochloride (MCA; LAP) (Millipore Sigma). These substrates target the β -glucosidase, alkaline
70 phosphatase, chitinase, and leucine aminopeptidase activities, respectively.

Incubations were performed at substrate-saturating concentrations (240 μ M final concentration) in the dark and at in situ temperatures. Incubation times were 2 h for estuarine samples and 24 h for offshore samples. Fluorescence was measured three to four times during each incubation using a Tecan Infinite 200 PRO microplate reader. Potential enzyme activities were calculated from the linear increase in fluorescence over time using MUF and MCA calibration standards.

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2.3 Statistical analysis

Differences in potential enzyme activities among initial, -20°C, and -80°C treatments were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($p < 0.05$). Statistical analyses were performed in JMP® software.

2 Results and discussion

80 Potential activities of four enzymes, leucine aminopeptidase (LAP), β -glucosidase (β -glu), N-acetyl- β -D-glucosaminidase (NAG), and alkaline phosphatase (AP), were measured immediately after sampling and after one month of storage at either -20°C or -80°C to evaluate whether extracellular enzyme activities can be retained on frozen samples for later analysis. In most cases, storage at -80°C yielded greater activity retention than storage at -20°C, although the magnitude of this effect varied among enzymes and sample types.

85 Both estuarine and offshore filters stored at -80°C showed high preservation of enzymatic activities (Fig. 1; Table 2). In estuarine filter samples, LAP and β -glu retained approximately 84% and 95% of their initial activities, respectively, while offshore filter samples showed little evidence of activity loss for LAP, β -glu, or NAG after storage. In contrast, AP exhibited greater sensitivity to freezer storage than the other enzymes tested. Most notably, AP activity on offshore filters declined by approximately 80% after storage at both -20°C and -80°C, whereas activity retention on estuarine filters remained
90 comparatively high, averaging approximately 80% of initial activity. Unlike the other enzymes examined, AP is a Zn-



containing metalloenzyme, and freeze-thaw processes may alter protein structure or disrupt enzyme-cofactor interactions, reducing catalytic activity. This sensitivity may have been partially mitigated in the particle-rich estuarine samples, where interactions with particles or organic matrices could have stabilized the enzyme during storage, resulting in higher activity retention than observed in offshore samples.

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Table 2. Potential enzyme activities (mean±SD; n=3) measured immediately after sampling and after one month of storage at -20°C or -80°C. Estuarine rates are in nmol L⁻¹ hr⁻¹; offshore rates are in pmol L⁻¹ hr⁻¹. Storage effects were assessed using one-way ANOVA with Tukey's post hoc tests. Different letters indicate significant differences among treatments (p < 0.05). LAP - leucine aminopeptidase; β-Glu - β-glucosidase; NAG - N-acetyl-β-D-glucosaminidase; AP - alkaline phosphatase.

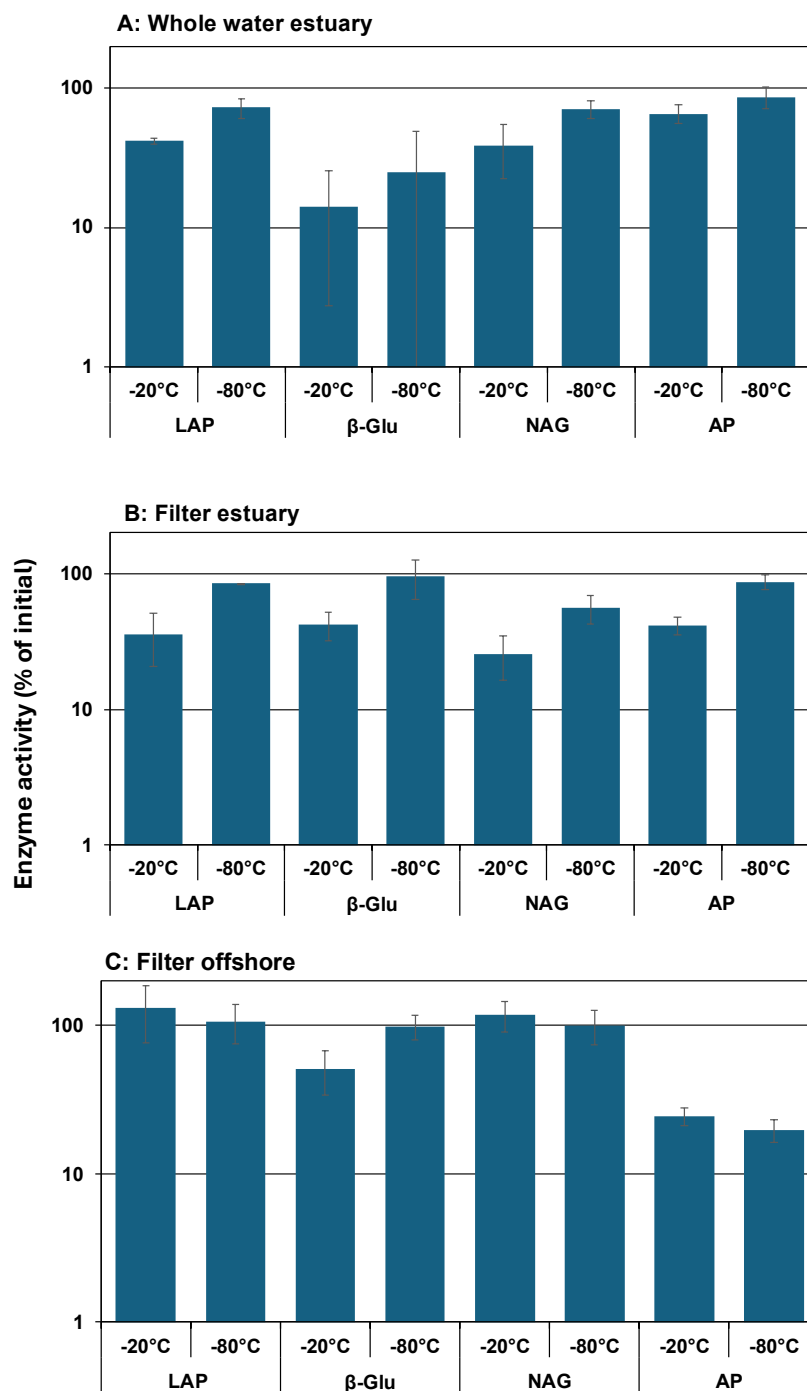
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Whole water estuary	Initial	1 month, -20°C	1 month, -80°C	p-value
LAP	205.1±15.6 (A)	85.3±1.9 (C)	147.8±14.5 (B)	<0.0001
β-Glu	76.6±30.5 (A)	10.4±9.9 (B)	19.1±21.0 (B)	0.02
NAG	14.3±0.8 (A)	5.4±2.0 (C)	10.2±1.6 (B)	0.001
AP	81.2±14.5 (A)	52.2±3.5 (B)	69.0±8.8 (AB)	0.03
Filter estuary				
LAP	507.5±15.1 (A)	181.9±77.0 (B)	428.4±14.8 (A)	0.0003
β-Glu	28.2±7.1	11.9±4.3	27.7±15.2	0.15
NAG	30.4±9.7 (A)	7.3±1.4 (B)	16.2±2.3 (AB)	0.008
AP	59.7±15.7 (A)	24.3±2.7 (B)	50.8±6.7 (B)	0.01
Filter offshore				
LAP	130.2±72.7	143.7±4.3	123.5±27.3	0.85
β-Glu	19.2±5.3	9.1±0.3	18.7±5.9	0.06
NAG	9.3±1.1	10.7±1.2	9.1±1.2	0.26
AP	175.2±21.7 (A)	42.3±0.5 (B)	33.9±2.4 (B)	<0.0001

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In contrast to filter samples, freezer storage of estuarine whole water was substantially less effective at preserving enzymatic activity. All four enzymes exhibited significant declines in activity after storage at both temperatures (Fig. 1; Table 2), suggesting that particle-associated enzymes may be better protected from freeze-thaw damage than dissolved enzymes present in the water column. During freezing, ice crystal formation concentrates salts and dissolved compounds within the remaining liquid phase, potentially altering enzyme conformation and reducing catalytic efficiency. Freeze-thaw cycles can further disrupt weak enzyme-substrate and enzyme-particle associations, particularly for dissolved enzymes that lack the protective environment provided by particles, biofilms, or cellular matrices. As a result, enzymes may partially denature or lose structural integrity during storage, leading to lower measured activities upon thawing. These effects are expected to be more pronounced in samples stored at -20°C than at -80°C (Chrost and Velimirov 1991), consistent with the generally higher activity retention in the estuarine whole water sample stored at -80°C.

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115 **Figure 1. Potential enzyme activities expressed as a percentage of activities measured on the day of sampling (initial rates; set to 100%). Bars represent mean values $\pm$ standard deviations ($n=3$). LAP - leucine aminopeptidase; β -Glu - β -glucosidase; NAG - N-acetyl- β -D-glucosaminidase; AP - alkaline phosphatase. The y-axis is shown on a logarithmic ($\log_{10}$) scale to facilitate visualization of activity changes.**



Overall, our results demonstrate that frozen storage, particularly at -80°C , preserves several ecologically relevant extracellular
120 enzyme activities on particulate samples without the need of preservatives or substrate additions before freezing as suggested
for whole water preservation (Chróst and Velimirov 1991; Christian and Karl 1995). While enzyme-specific limitations
remain, most notably for AP, archived filters provide a promising tool for coupling hydrolytic enzyme measurements with
omics analyses routinely conducted on frozen material. Generating molecular and enzymatic measurements from the same
filters strengthens the connection between microbial community composition, functional potential, and biogeochemical rates
125 in marine ecosystems. Such an approach offers a practical framework for linking microbial physiology with ecosystem
processes. This work aligns closely with emerging international efforts within BioGeoSCAPES to integrate microbiological
and biogeochemical observations with numerical models to improve our understanding of marine microbial biogeochemistry
(Saito et al. 2024).

Author contributions

130 KZ designed the experiments, and JL and LW carried them out. KZ prepared the manuscript with contributions from all co-
authors.

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